Attorney's Docket No. 024916-011

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	
Anthony Jevnikar et al.	Group Art Unit: 1644
Application No.: 10/005,073	Examiner: GERALD R EWOLDT
Filed: December 7, 2001	Confirmation No.: 8806
For: METHODS AND PRODUCTS FOR CONTROLLING THE IMMUNE RESPONSES IN MAMMALS	

DECLARATION OF ANTHONY M. JEVNIKAR

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Anthony M. Jevnikar, do hereby declare and say as follows:

1. I am a co-inventor named in the above identified application.

2. I have read and understood the Office Action mailed on October 8, 2004 (the "Office Action").

3. In the Office Action, the Examiner has rejected claims 52, 59-61, 63, 69-91 and 95 "under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention." OFFICE ACTION at 2.

The Examiner has argued that the claimed invention is not adequately enabled because ". . . results in mouse models do not correlate with results in humans. Whereas tolerance has been repeatedly induced in mice, the identical/equivalent methods have not worked in humans". OFFICE ACTION at 2.

This rejection was discussed during an Examiner interview on January 18, 2005 in which I was present. During the interview, the Examiner had indicated that three documents referred to in the last Office Action dated February 10, 2004, namely, Marketletter 1999; Goodnow 2001; and WO 02/53092 supported the notion that it has been repeatedly shown that results in mouse models do not correlate with results in humans. Furthermore, the Examiner asserted that there is in fact a lack of efficacy and indeed, danger, of administering antigens to humans in attempts to induce tolerance.

Firstly, the Marketletter 1999 Newsletter appears to be a newspaper publication and may contain errors as it is not a scientific publication and is thus not peer reviewed. For this reason alone, I would not consider this document to be a reliable scientific document. In any event, this newsletter clearly states that Colloral had failed in Phase III development not because it had no effect or because it posed any danger to human health, but rather because the effect it did have in human clinical trials was not statistically significant enough to warrant further spending on late phase clinical testing.

The document by Goodnow et al., is an overview of self-tolerance pathways and is not a description of a specific experiment that is designed to induce tolerance. In fact, the last sentence of the abstract that the Examiner cited is directed to the strategies noted earlier in the abstract which are not the same strategies as currently claimed and

described in the subject patent application for which I am a co-inventor. The statement in Goodnow et al. regarding the unpredictability, is in fact directed against the use and mechanism of action of corticosteroids.

WO 02/053092 discloses a method of inducing immune tolerance to plaque associated molecules such as LDL, beta-2-GPI and HSP in humans. The Examiner refers to page 23 of the description to assert that the inventors of the '092 PCT publication conclude that "oral and mucosal tolerance cannot be deduced from antigenic activity in conventional immunization, or even *in vitro* results and must result from extensive empirical experimentation". It should be noted that this conclusion in the '092 PCT publication is with respect to a summarized description of prior art that does not involve any oral antigen presentation by the oral administration of plant material that contains the antigen as is the case in the presently claimed invention.

Despite the prior art summary discussion on page 23 of the '092 PCT publication, the inventors of the '092 PCT publication proceeded to overcome the described short coming of the prior art with their invention. The '092 PCT publication ultimately shows that immune tolerance can be accomplished.

It is clear that these documents do not address the method of the subject invention for which I am a co-inventor which is a method for suppressing or reducing an immune response by administration of an antigen as is expressed and contained in a plant material.

I further disagree with the Examiner's statement that oral tolerance has never before been successfully demonstrated in humans. Oral tolerance to protein antigens

has in fact been demonstrated in humans after antigen ingestion. Attached hereto as Exhibit A is a copy of a paper by Husby et al., Oral Tolerance in Humans, The American Association of Immunologists, 1994, pp. 4663-4670, that clearly demonstrates oral tolerance in humans. More specifically, this work demonstrates the suppression of peripheral T cell responses to the protein antigens KLH, after oral administration of KLH.

Oral tolerance has also been demonstrated by the administration of Type I collagen to autoimmune patients with Systemic Sclerosis. This was demonstrated to induce significant reductions in levels of INFγ and IL-10 in stimulated peripheral blood lymphocyte culture supernatants, indicating that T cell immunity to collagen was decreased by oral collagen administration. This study is described in McKown et al., Arthritis & Rheumatism, Vol. 43, No.5, May 2000, pp. 1054-1061 (Exhibit B).

These two representative references support the fact that oral tolerance can be induced in humans and support the assertion that oral tolerance can be useful therapeutically.

4. A NIH clinical trial has been recently concluded which involved orally administering insulin to children at high risk for developing Type I diabetes (Diabetes Prevention Trial-1, DPT-1). Unfortunately, detailed results of this study have not yet been published. However a summary of the results in a subset of children with the highest levels of anti-islet cell antibodies that received oral insulin has been personally communicated to me by Dr. Noel MacLaren, an international expert in autoimmune

diabetes and participating investigator in the DPT-1 trial. Dr. MacLaren is currently a Professor of Pediatrics in the Department of Pediatrics at the Weill-Cornell Medical School. Dr. MacLaren was formerly at the University of Florida, College of Medicine. The oral administration of insulin significantly reduced the development of diabetes in the 5 years of the study in the subset of children with the highest levels of antibody to insulin. This study demonstrates the benefit to those at the highest risk for the disease. Additionally, a publication by MacLaren et al (New York Academy of Science, in press, 2005) demonstrates oral insulin also has a beneficial effect through oral tolerance in patients with established Type I diabetes.

Again, the results of these studies further demonstrate that oral tolerance can be induced in humans.

5. The Examiner has stated in the Office Action that the previous declarations that I have executed and were previously submitted in response to the Office Action mailed on February 10, 2004, admit that the experimental evidence provided therein was "unexpected" as thus seen as "an admission of the unexpected nature of the instant inventions". This is not the case. The use of the term "unexpected" in those declarations was merely to state that until the development of the present invention, one of skill in the art did not contemplate and thus would have not expected that an antigen could be successfully produced and administered in the same plant tissue which could then be orally administered. The presently claimed invention provides such a method. Accordingly, the presently claimed invention provides for

"unexpected" advantages over the prior art that were not previously realized; such unexpected effects were due to the fact that plant-derived antigens could be expressed in plants and administered orally such that the antigens are not adversely affected by digestion and thus can be more effective *in vivo*.

Thus, the plant could be used as both the expression vehicle and mode of administration for the antigen and this was never before contemplated.

6. The Examiner's assertion that the results provided in my two earlier executed declarations were just as or (more) likely due to "the specific animal model; the specific disease model; the specific antigen; or the specific transgenic plant species expressing the antigen" is unfounded. The experimental set up described in my two earlier declarations demonstrate favorable and beneficial results in mice. The experimental set-up is a credible *in vivo* model that is often used by those of skill in the art. The results provided in my earlier declarations clearly support the instant claims.

7. The Examiner has asserted that the animal data of record is not sufficient to satisfy the enablement requirement. However, as the Examiner stated during the above-noted interview, human *in vivo* data is not in fact required for enablement under United States law.

The animal data which has been provided in the present application would clearly be understood by those of skill in the art to support the use of the claimed methods and compositions in humans. In the case of both mice and humans, immune responses in

lymphocytes upon *in vitro* challenge to a specific protein is similarly attenuated or changed following oral administration of the protein. No qualitative or quantitative differences are found in the pattern of cytokines released or T cell activity and so mice and humans share a common biological response to oral protein antigens. Importantly, the NIH data in diabetes represents the clearest link yet between an autoimmune mouse disease model and autoimmune human disease, as both respond to an endogenous autoantigen protein that causes spontaneous diabetes.

8. The Examiner has rejected claims 52, 59-61, 63, 69-91 and 95 under 35 U.S.C. 103(a) as being unpatentable over WO 92/07581 in view of U.S. Patent No. 5,484,719 ("the '719 Patent"). *See* OFFICE ACTION at 3-4. WO 92/07581 discloses a method of suppressing an immune response by administering cell extracts from a donor. This reference does not teach any type of oral administration involving the use of any plant. The '719 Patent discloses expressing viral, bacterial or fungal antigens in a plant as a method of vaccination against harmful pathogens. This invention would only be useful for vaccination against pathogens which is a completely different use and thus method to that presently claimed. The presently claimed invention is not directed to vaccination to prevent any type of infectious agent. Thus, the '719 patent is not relevant to the presently claimed invention. Vaccination to pathogens using oral administration of pathogen antigens represents completely different mechanisms for beneficial effect to that of immune tolerance to the endogenous proteins involved in autoimmunity. One of skill in the art would not be motivated to combine the teachings of

these two references and, even if combined, it does not permit one skilled in the art to arrive at the methods claimed in the subject patent application.

9. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: February 8, 2005

A. M. Jevnikar

Anthony M. Jevnikar

Oral Tolerance in Humans

T Cell but Not B Cell Tolerance After Antigen Feeding[1]

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The purpose of this study was to investigate whether oral tolerance, defined as Ag-specific immunologic unresponsiveness after Ag feeding, could be induced in humans after prolonged Ag ingestion. Eight adult volunteers ingested a total dose of 0.5 g of keyhole limpet hemocyanin (KLH) followed by subcutaneous immunization with KLH. Eight controls received only the subcutaneous immunization. In the group fed KLH, there was a significant reduction in KLH-specific T cell proliferation ($p = 0.04$) and delayed skin test responses ($p = 0.07$) to KLH. KLH ingestion alone did not induce significant levels of Abs in either serum or secretions. However, after the subsequent subcutaneous immunization, the number of circulating IgG and IgM anti-KLH-producing cells, the titers of serum IgG, IgA, and IgM anti-KLH Abs, and the titers of IgA anti-KLH Abs in saliva and intestinal secretions were significantly greater in the KLH-fed group than in the nonfed group. We conclude that KLH feeding induced systemic T cell tolerance, but B cell priming, at both systemic and mucosal sites. These studies support the concept of using Ag feeding as a treatment for certain immune-mediated diseases. *Journal of Immunology,* 1994, 152: 4663.

The environmental Ags from food and microbial flora are in constant contact with mucosal surfaces and provide a continuous stimulus for the entire immune system. Although a common result of such stimulation is the induction of mucosal and systemic immunity, an alternative outcome is a state of unresponsiveness or tolerance (1, 2). The term oral tolerance refers to a state of systemic unresponsiveness to parenteral immunization that is induced by previous Ag feeding. Oral tolerance of both humoral and cellular immunity has been convincingly demonstrated in rodents fed a wide variety of Ag types (3–8). In several experimental autoimmune diseases, such as experimental allergic encephalomyelitis (9–12), collagen-induced arthritis (13, 14), and experimental autoimmune uveitis (15), autoantigen feeding has blocked induction of or ameliorated established disease. In some of these models, such Ag feeding has been found to induce $CD8^+$ T cells that secrete the cytokine TGF-β upon Ag reexposure in vivo (16, 17). These results have prompted an interest in the feeding of autoantigens as a therapy for human autoimmune diseases (18, 19). However, attempts to induce oral tolerance in some species, such as rabbits, have been unsuccesful (20), and it is unclear whether this approach could be effective in humans, in whom the existence of oral tolerance has not been clearly demonstrated, although suggested (21, 22).

The purpose of this study was to determine whether Ag feeding of humans induces oral tolerance of either the T cell or B cell compartment. We used KLH,[4] a potent systemic immunogen, because it is a novel Ag to most individuals, and it has been used safely in humans to assess immunocompetence. The strategy was to feed KLH to a group of volunteers; this group and another group not fed KLH were then parenterally immunized, and the ensuing systemic and mucosal immune responses were compared. T cell responses were assessed by a proliferation assay and by skin test reactivity to purified KLH. B cell responses were assessed by ELISA for IgM, IgG, and IgA anti-KLH

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[4] Abbreviations used in this paper: KLH, keyhole limpet hemocyanin; ELISPOT, enzyme-linked immunospot; SI, stimulation index; NC, nitrocellulose; SFC, spot-forming cell; EU, endotoxin units.

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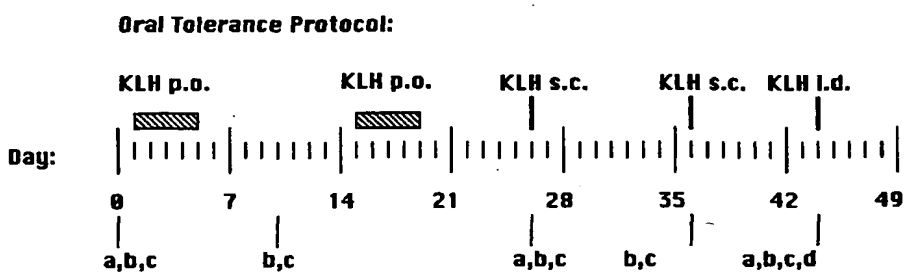

FIGURE 1. Protocol of feeding, parenteral immunization, and testing used in this project. KLH p.o. = 50 mg of KLH was administered in gelatin capsules per os daily; KLH s.c. = 100 μg of KLH injected s.c.; KLH i.d. = 10 μg of KLH injected intradermally as a skin test. Samples for testing were obtained as shown: *a*, peripheral blood cells for ELISPOT and proliferation assays; *b*, serum for ELISA; *c*, intestinal secretions and saliva for ELISA; *d*, delayed skin test responses.

Abs in serum, for IgA anti-KLH Abs in secretions, and by the ELISPOT technique for Ab-producing cells in peripheral blood.

Materials and Methods

Volunteers

A total of 16 healthy volunteers were recruited for the study; 8 of the volunteers (mean age 28 yr, range 23–37; 6 males and 2 females) took part in the study as the experimental group. The other 8 subjects were included in the control group (mean age 27 yr, range 21–36; 7 males and 1 female). The study was approved by the Human Use Committee for the University of Alabama at Birmingham. Informed consent was obtained from each subject before participation.

Experimental design

Fasting volunteers of the experimental group ingested 50 mg of KLH in gelatin capsules on days 1 to 5 and days 15 to 19. They were then immunized s.c. with 100 μg KLH on day 25 and boosted with the same dose on day 36. The control group underwent the parenteral immunization only (Fig. 1). Samples of blood, saliva, and intestinal secretions were obtained at intervals for assessment of immunity. Blood samples were taken by venipuncture before the start (day 0) and on days 10, 25, 36, and 44 of the study. Secretions were obtained before (day 0) and on days 25 and 44 of the study. An intradermal skin test with KLH (10 μg) was applied on day 44 and read on days 45 and 46.

Keyhole limpet hemocyanin

KLH as a freeze-dried powder was purchased from Sigma Chemical Co. (St. Louis, MO). For oral use, 50 mg of this preparation was packed into gelatin capsules, which were filled with lactose. For parenteral use, this preparation was dissolved in pyrogen-free saline and passed two times through a polymyxin-agarose column (Boehringer-Mannheim, Mannheim, Germany) at a concentration of 4 mg/ml. This treatment diminished the endotoxin content of the KLH preparation from approximately 1100 EU/ml to below 10 EU/ml as confirmed by Limulus assay (Whittaker, Walkersville, MD). The preparation was filter-sterilized, aliquoted in pyrogen-free saline plus 0.001% merthiolate, and stored at 4°C until use.

Cell isolation and purification

Heparinized blood was diluted 1:2 into Dulbecco's PBS (GIBCO BRL, Grand Island, NY), and the mononuclear cells were isolated by gradient centrifugation over Ficoll-sodium diatrizoate (Organon Teknika, Durham, NC) in 15 ml tubes at 2000 rpm for 20 min, the serum-Ficoll interface was collected and the cells were washed in Dulbecco's PBS followed by RPMI 1640 (GIBCO BRL), supplemented with 10% FCS (Irvine Scientific, Santa Ana, CA), 100 U penicillin/ml and 100 μg streptomycin/ml. The cells were counted, adjusted to 5×10^6/ml, and used directly in the ELISPOT assay. A T cell-enriched fraction was prepared by rosetting with 2-amino-ethylisothiuronium bromide-treated SRBCs, prepared as described (23) except that the rosetting was allowed to take place overnight on ice. Cells at the plasma-Ficoll interface were removed (E^- cells) and the T cell-enriched fraction (E^+ cells) was prepared by lysis of the SRBCs (24). The E^+ fraction typically contained 60 to 80% of the total number of cells. The E^- cells contained 40 to 50% monocytes as measured by the esterase stain for monocytes/macrophages (25). E^- cells were irradiated at 3000 rad before use as APCs in culture.

T cell proliferation assay

Sterile 96-well microtiter plates (Costar, Cambridge, MA) were used for cell culture. Quadruplicate wells were prepared with adherent APCs by the incubation of 100 μl of 10^6 E^- cells/ml for 2 h. The wells were washed once with medium and the T cell-enriched (E^+) cells were added at 2×10^6 cells/mL. Replicate wells received KLH (10 μg/ml), PHA (Sigma Chemical Co., 2 μg/ml) as a positive control, or medium alone as a negative control. The plates were incubated at 37°C and 5% CO_2 for 2 days for PHA responses and 5 days for KLH-specific responses. The wells were then pulsed with [^{3}H]thymidine (Amersham, Arlington Heights, IL) at 0.5 μCi/well for 6 h and harvested on nylon filters; cpm were measured with a liquid scintillation counter. The results were expressed both as a stimulation index (SI), i.e., the ratio of the mean KLH-stimulated cpm divided by the mean unstimulated cpm and as Δ cpm, i.e., the mean KLH-stimulated cpm minus the mean unstimulated cpm.

KLH-stimulated T cell cytokine production

T cells and APCs were cultured with KLH (10 μg/ml) in wells of 24-well culture plates at 2×10^6 cells/well. After incubation at 37°C and 5% CO_2 for 48 h, the supernatants were harvested and frozen at −20°C until assay. The IFN-γ assay was performed using the WEHI 279 cell line, whose growth is inhibited by IFN-γ (26). TGF-β was measured in a bioassay using the cell line CH-1CAB, whose growth is inhibited by TGF-β (27). A standard curve was constructed for each cytokine and the values for the experimental samples interpolated. In both assays, cell growth was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide method as described (25).

Delayed skin test reactivity to KLH

Endotoxin-free KLH (10 μg in 100 ml) was injected intradermally at the flexor surface of the forearm using pyrogen-free saline as the control. Induration was measured in mm at 24 h and 48 h by the same observer. A reaction was considered positive if any measurable induration was present.

Enumeration of Ab (spot)-forming cells (SFCs) by ELISPOT

To determine the numbers of IgG, IgA, and IgM anti-KLH-producing cells, the ELISPOT assay was used as described previously (29). Nitrocellulose (NC) 96-well microtiter plates (Millipore Corp., Bedford, MA) were incubated with KLH at a concentration of 20 μg/ml in PBS overnight at room temperature. For the enumeration of isotype-specific Ig-producing cells, the wells were incubated with $F(ab')_2$ fragments of anti-IgG, anti-IgM, or anti-IgA Abs (Jackson Immunoresearch Labs, West Grove, PA). Nonspecific protein binding to the NC was blocked by incubation for 2 h with PBS + 10% FCS. The mononuclear cell fraction from the heparinized blood sample was isolated as described above. 100 μl of cell suspension was immediately added in duplicate onto the plate at a range of concentrations from 10^5 to 5×10^6 cells/ml for anti-KLH Ab-producing cells and from 10^4 to 10^6 cells/ml for total Ig-producing cells. After incubation at 37°C for 3 h, the wells were washed three times with PBS and three times with PBS containing 0.05% Tween (Sigma Chemical Co.), and incubated overnight at 4°C with 100 μl of biotin-labeled goat $F(ab')_2$ anti-IgG, -IgA, and -IgM (Tago, Burlingame, CA) diluted 1:750 in PBS-Tween + 1% FCS. The wells were blotted dry, washed, and developed with Extravidin-alkaline phosphatase (Sigma Chemical Co.) followed by the chromogen substrate. The substrate was prepared by mixing 15 mg 5-bromo-4-chloro-3-indolylphosphate toluidine salt (Bio-Rad Labs, Richmond, CA) and 30 mg p-nitroblue tetrazolium chloride (Bio-Rad Labs), separately dissolved in 1 ml dimethylformamide, and adding the mixture to 100 ml 0.1 M $NaHCO_3$ + 1 mM $MgCl_2$, pH 9.8. The NC plate was blotted dry and the wells were exposed to the chromogen substrate; blue spots appeared, usually within 30 min, where a positive reaction had occurred. When the spots had reached maximal intensity, the plate was rinsed with tap water and allowed to dry. The spots were enumerated under a stereomicroscope at 40-fold magnification. The active synthesis of Ab was confirmed by the incubation of the cells with cycloheximide (25 mg/ml for 2 h), which resulted in 70% to 100% inhibition of SFCs.

Intestinal and salivary secretions

Intestinal secretions and saliva samples were obtained from eight subjects in the fed group and from four subjects in the control group. The intestinal secretion samples were obtained with the use of a polyethylene glycol salt solution Colyte® (Reed & Carnrick, Piscataway, NJ), as described previously (30). Saliva was collected as unstimulated whole saliva by having the subject drool into a centrifuge tube placed in ice. Unstimulated parotid saliva was collected using a Schaefer cup placed over the parotid duct (31). The saliva samples were centrifuged at 10,000 rpm for 3 min in a microfuge to remove debris.

Measurement of Ab by ELISA

Flat bottom polystyrene microtiter plates (Titertek, Flow Labs., McLean, VA) were coated with KLH (10 μg/ml of PBS), blocked with 5% FCS in PBS for 2 h, and washed three times with PBS-Tween. For analysis of serum samples, serum was diluted 1:250 and 1:1000 in PBS-Tween + 1% FCS. For analysis of saliva, the samples were diluted 1:10 and 1:50 in PBS-Tween containing 1% FCS. The plates were incubated overnight at room temperature. For analysis of intestinal secretions, the samples were diluted 1:4 and 1:40 in PBS-Tween + 5% FCS and incubated overnight at 4°C. The washed plates were incubated with biotinylated goat $F(ab')_2$ anti-IgG (1:8000), anti-IgA (1:1000), or anti-IgM (1:1000) (Tago) for 4 h at 37°C, followed by Extravidin-alkaline phosphatase (1:2000) for 2 h and then developed with para-nitrophenylphosphate substrate (Sigma Chemical Co.). The absorbance was read at 405 nm in a V_{max} kinetic microplate reader (Molecular Devices, Palo Alto, CA). A high titer serum was used as a reference standard and defined to contain 1000 U/ml IgG, 100 U/ml IgA, and 100 U/ml IgM anti-KLH Ab, based on their relative titer in serum. The absorbances of the samples were analyzed and converted into ELISA U/ml by using a computer program based on a four-parameter logistic model. Two control sera were included in each plate; based on the results of these control sera, the intra-assay variation was 3 to 7%, and the interassay variation of anti-KLH Abs was 22% to 26% for IgG, 15% to 26% for IgA, and 14% to 16% for IgM ($n = 26$).

Samples of sera from days 0, 25, and 44 were also tested for Ab to tetanus toxoid and bovine gammaglobulin. Serially diluted sera were tested in Ag-specific ELISA and the Ab concentrations were expressed as ng/ml by referring to calibration curves for each Ig (Ig) isotype, constructed by assaying wells on the same plates coated with anti-Ig isotype Abs and calibrated serum standards.

Total Ig levels in secretions

The amount of IgG, IgA, and IgM in intestinal secretions was assessed by ELISA. Microtiter plates (Titertek, Flow Labs) were coated with affinity-purified goat $F(ab')_2$ anti-IgG (2.5 μg/ml), anti-IgA (5 μg/ml), or anti-IgM (2.5 μg/ml) in PBS (all from Jackson Immunoresearch Labs), blocked with 5% FCS in PBS, and washed three times. The samples were added at a dilution of 1:400 and 1:4000 in PBS-Tween + 5% FCS and incubated overnight at 4°C. The reference standards used were purified colostral IgA (2.9 g/l, Ref. 32) and the Monitrol standard (Baxter, McGaw Park, IL) for IgG (9.9 g/l) and for IgM (0.9 g/l). The plates were washed and incubated with biotin-labeled $F(ab')_2$ anti-IgG, anti-IgA, or anti-IgM (Tago), and developed as above for the Ab determinations.

Statistical analysis

Nonparametric statistical analysis was used. Comparison between groups was performed with the Mann-Whitney U-test for unpaired samples. Comparison within groups was done with the Wilcoxon/Pratt test for comparing two samples and the Friedman test for comparing several samples. The level of significance was chosen as $p < 0.05$.

Results

Induction of T cell tolerance by feeding

T cell proliferation assay. Ag-specific T cell proliferation was assessed before immunization (day 0), after the oral immunization (day 25), and after the parenteral immunization (day 44) (Fig. 2). The SI in both groups was low before the immunization. After the oral immunization, the SI for the KLH-fed group rose moderately but significantly ($p = 0.047$) and at this time point was also higher than the preimmunization value of the control group ($p = 0.007$). However, after the parenteral immunization, the SI in the KLH-fed group was significantly lower than in the nonfed group ($p = 0.04$) (Fig. 2*A*). Similar results were found when the data are expressed as Δ cpm, with an increase after the feeding in five of eight subjects, followed by a reduction to baseline levels after the parenteral immunization (Fig. 2*B*). Mean PHA-stimulated T cell proliferation of the KLH-fed group did not change significantly in the KLH-fed vs control groups either after the feeding or after the parenteral immunization, nor in the KLH-fed group before feeding, after feeding, or after parenteral immunization (data not shown).

Delayed skin test response. Intradermal skin testing with KLH was done on day 44 (Table I). At 24 h, only one of eight subjects in the KLH-fed group had a positive reaction, whereas seven of eight in the control group were positive ($p = 0.007$). The same pattern was seen at 48 h, when zero of eight in the fed group were positive vs five of eight in the control group ($p = 0.038$).

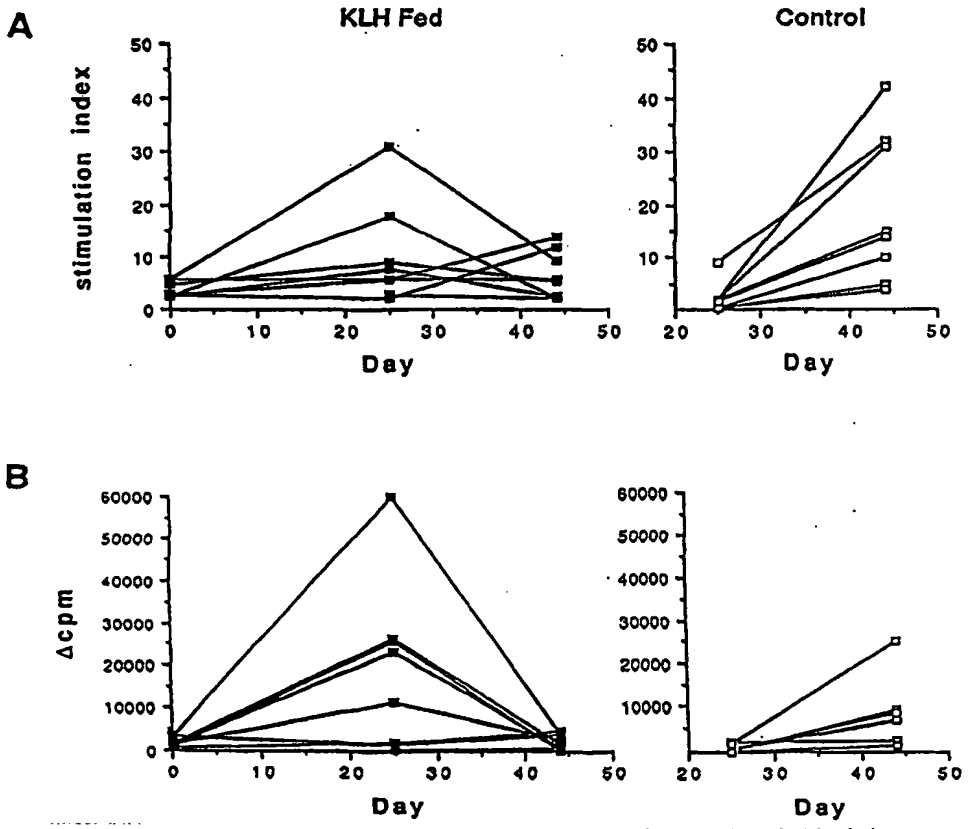

FIGURE 2. KLH-induced proliferation of peripheral blood T cells. The data from each individual shown at day 0 before the study, at day 25 after the oral feeding, and at day 44 after subcutaneous immunization. *A*, stimulation index; *B*, Δ cpm.

Priming of the systemic B cell response by KLH feeding

Anti-KLH SFCs in peripheral blood. Virtually no anti-KLH SFCs were identified in the blood either before or after the KLH feeding. However, IgG, IgA, and IgM anti-KLH SFCs were detected after the parenteral priming and booster doses (Fig. 3). IgG and IgM anti-KLH-secreting cells were significantly greater in number in the KLH-fed group than in the control group only after the parenteral priming on day 36 ($p = 0.028$ and $p = 0.021$, respectively). There were no statistically significant differences on day 44.

Serum anti-KLH Abs. Feeding with KLH did not produce any detectable serum levels of anti-KLH Abs of any isotype above the background level (Fig. 4). However, KLH feeding did result in the priming of B cells, as demonstrated by a significantly higher serum IgG anti-KLH (Fig. 4*A*) in the KLH-fed group as compared with controls following the parenteral priming and booster immunization ($p < 0.05$). Both IgA and IgM anti-KLH in the KLH-fed group rose sharply and significantly ($p < 0.01$ for both; Figs. 4, *B* and *C*) after the parenteral priming, but were not significantly different from the control group after the parenteral booster.

Serum Abs against a food Ag, bovine gammaglobulin, as well as against tetanus toxoid, were measured in samples obtained at days 0, 25, and 44. No change in mean Ab titer in either the KLH-fed or the control group was found for either of these Ags (data not shown).

Priming of secretory Abs by KLH feeding

Anti-KLH Ab levels in salivary secretions. The IgA anti-KLH levels in both whole saliva (Fig. 5*A*) and parotid saliva (data not shown) of the KLH-fed group did not increase significantly after the feeding alone, but did increase after the subcutaneous immunization with KLH (day 44) ($p < 0.005$ for whole saliva and $p < 0.015$ for parotid saliva).

Anti-KLH Ab levels in intestinal secretions. No IgG or IgM anti-KLH was detected in the intestinal secretions. IgA anti-KLH levels in intestinal secretions were low, and although they were enhanced after the KLH feeding, this increase was not statistically significant. However, secretory IgA anti-KLH was increased in the KLH-fed group after the parenteral immunization (Fig. 5*B*) when compared with their own day 0 base line levels ($p = 0.016$).

KLH-stimulated cytokine secretion. On days 0, 25, and 44, T cells were cultured with APC and KLH (10 μg/ml) for

Table I. *Delayed skin test response to KLH*[a]

Group	24 h		48 h	
	Mean (range)	Number of positive/total	Mean (range)	Number of positive/total
KLH-fed	1.2 (0–10)	1/8	0 (0–0)	0/8
Controls	11.9 (0–23)	7/8	6.6 (0–20)	5/8
p-value	0.007		0.038	

[a] KLH (10 μg) was injected intracutaneously and the reaction measured as induration (mm) at 24 and 48 h after the injection.

48 h. Supernatants of those cultures were collected and tested for IFN-γ and TGF-β by bioassay. IFN-γ was not detected in any supernatant. TGF-β was detected in a small number of culture supernatants, but there was no relationship to KLH feeding or immunization (data not shown).

Discussion

The present study demonstrates that oral tolerance can be induced in humans; however, with the Ag, dosage, and immunization schedule used, tolerance was limited to the T cell compartment, as demonstrated by a reduction in Ag-specific T cell proliferation and markedly diminished delayed skin test reactivity. Although no anti-KLH-secreting cells or Abs were detected in blood or secretions after the oral feeding, the KLH feeding clearly had an impact on the immune system in that it primed B cells in systemic as well as secretory sites for a greater response upon parenteral immunization. Because the dose, frequency, and type of Ag are known to have a profound influence on the induction of oral tolerance in experimental animals (7, 8), it is possible that varying one or more of these parameters might induce tolerance in B cells in humans as well.

The purpose of this study was to determine whether oral tolerance to protein Ag existed in humans; a detailed examination of the Ag specificity of the tolerance was not planned as a part of the study because it is well established in experimental animals that oral tolerance to proteins is Ag-specific. However, the absence of any effect of KLH feeding on polyclonal T cell proliferation and on serum Ab titers to two common Ags does suggest that oral tolerance is Ag specific in humans as well.

Tolerance of T cells but not B cells has been previously identified after feeding (33, 34) and injecting (35–37) low doses of Ag to animals. A recent study performed in transgenic mice showed that the small amounts of autoantigen released spontaneously in vivo rendered the animals' T cells but not B cells tolerant (38). The B cell system can be tolerized by Ag feeding, but generally requires larger amounts of Ag (4–6). Sensitivity to tolerance induction also varies among T cell subsets. $CD4^+$ T cells can be divided into two major subgroups based on their production of cytokines. $CD4^+$ Th1 cells produce IL-2 and IFN-γ and mediate delayed hypersensitivity. $CD4^+$ Th2 cells produce IL-4, IL-5, IL-6, IL-10 and provide help for B cell

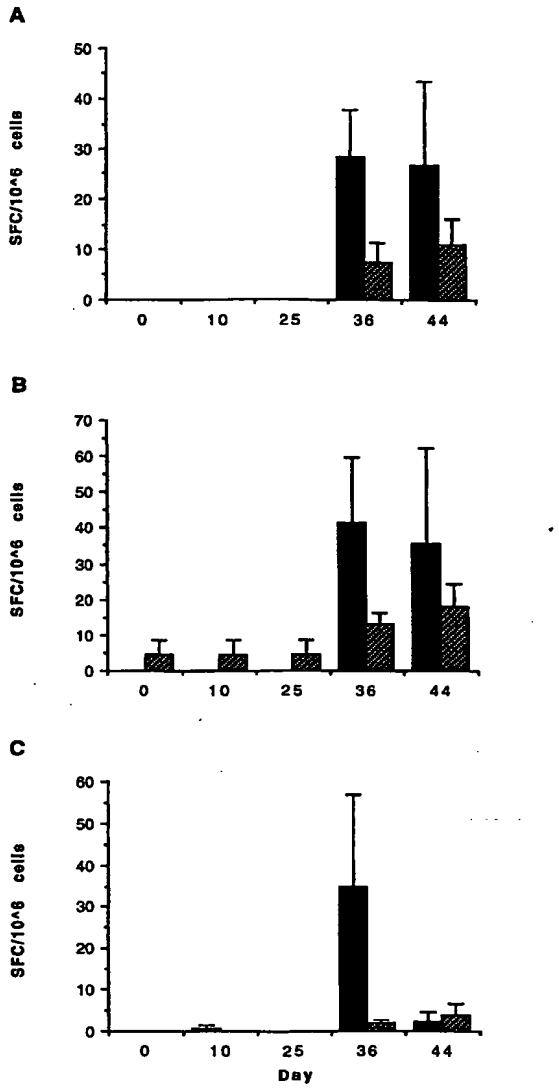

FIGURE 3. Circulating anti-KLH secreting cells (SFC)/10^6 PBLs of the (*A*) IgG, (*B*) IgA, and (*C*) IgM isotype before and after oral feeding and parenteral immunization with KLH. Solid bars denote the KLH-fed group and lined bars the control group. The data for the control group at days 0, 10, and 25 represent the preimmunization value. Data are shown as geometric means and SE.

Ab responses (39). Parenteral injection of soluble protein Ags into mice tolerized Th1 but not Th2 cells (40); the hyporesponsiveness in Th1 cells was mediated by IL-4 production by Th2 cells (41). There seems to be a gradient of sensitivity to tolerance induction, with Th1 cells > Th2 cells > B cells. Although the existence of the Th1/Th2 subsets is not as well established in humans as compared with mice, the present data are consistent with this paradigm. KLH-specific T cell proliferation and skin test responses are mainly Th1 functions, and these were significantly inhibited by KLH feeding. The B cell priming that

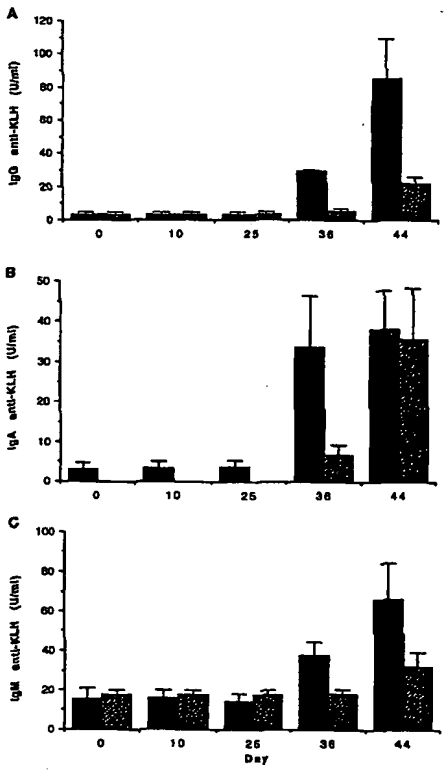

FIGURE 4. Serum Abs to KLH. Anti-KLH of (*A*) the IgG, (*B*) the IgA, and (*C*) the IgM isotype, obtained before the start of the study (day 0), after one period of KLH feeding (day 10), after two periods of KLH feeding (day 25), after parenteral priming (day 36) and after parenteral booster (day 44). The KLH-fed group is denoted by solid bars and the control group by lined bars. For the control group the results presented at days 0, 10, and 25 represent the preimmunization value. Bars represent geometric means and SE.

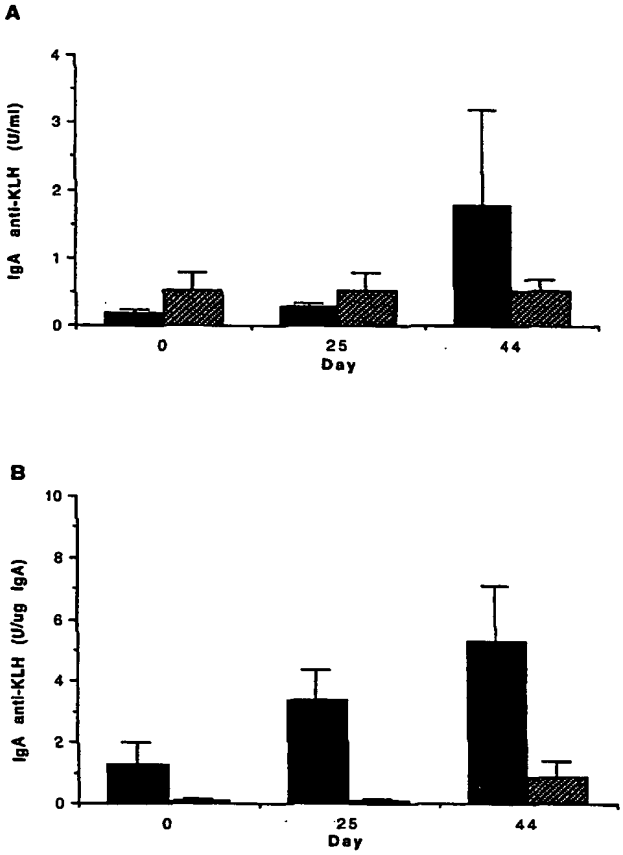

FIGURE 5. IgA anti-KLH Abs in secretions: (*A*) in whole saliva and (*B*) in intestinal secretions. Solid bars denote KLH-fed group and lined bars denote control group (four subjects only). For the control group, the results from days 0 and 25 represent the preimmunization value. Data are shown as geometric means and SE.

occurred from the KLH feeding is consistent with a lesser effect of the feeding on Th2 helper cells and B cells. The lack of any Ab production from the feeding alone could result from the dose of Ag being insufficient to trigger Th2 cells or KLH feeding having some inhibitory effect on Th2 cell function.

Tolerance may occur by a number of mechanisms, including clonal deletion, clonal anergy, or suppression. With regard to orally induced tolerance, local and systemic suppressor T cell circuits may be particularly important (5, 9, 42–44). Antigen feeding can generate Ag-specific suppressor T cells in the Peyer's patches of mice, and these T cells later populate systemic lymphoid tissues such as the spleen (42). However, even after suppressor T cells can no longer be identified, the animals remain unresponsive to the fed Ag (43), suggesting the presence of an additional mechanism such as clonal anergy. Ag feeding has been reported to stimulate $CD8^+$ T cells that release the immune-inhibitory cytokine TGF-β upon restimulation with Ag (16, 17). Accordingly, peripheral blood T cells from KLH-fed and control subjects were restimulated with KLH plus APCs in vitro. TGF-β was detected in a small number of samples, but its presence was inconsistent and had no relationship to either the feeding or parenteral immunization with KLH. These data do not exclude a possible role for TGF-β-producing cells, in that the relevant cells may not circulate in sufficient numbers to be detected. Studies detecting them have used lymphoid and other tissues (16, 17), not circulating cells. The role of this or other cytokines, such as IL-4, in oral tolerance remains to be defined.

Although the cellular and molecular mechanisms for the induction of oral tolerance in humans are unknown, oral tolerance may represent an important immunoregulatory process that limits immune response to innocuous food Ags. Certainly, humans ingest food Ags daily in quantities that should result in tolerance, and a small fraction is known to be absorbed into the circulation (45). When

adults with low levels of Ab to BSA were immunized by either ingestion or parenteral injection of BSA, they did not develop an Ab response (21), which may represent a form of oral tolerance to this food Ag. Despite the apparent occurrence of tolerance to food Ags, secretory and serum Abs to them are readily detectable in humans (46–48). The gradient of sensitivity of T cell subsets and B cells discussed above may explain this apparent paradox. Prolonged ingestion of Ag may be sufficient to prime B cells and Th2 cells sufficiently for the production of low levels of Ab.

Despite the large amount of secretory IgA Ab produced daily, it has been difficult to induce secretory IgA responses at mucosal surfaces in any species, including humans, by oral immunization with soluble or nonviable particulate Ags (49). The present results illustrate this, in that even 10 days of Ag feeding did not result in significant Ab production in saliva or intestinal secretions. Thus, these results have implications relative to strategies for oral vaccines. One prediction is that oral-parenteral combinations may be more effective for immunization in humans than the oral route alone. Another possibility is that protein Ags given orally will require mucosal adjuvants to prevent induction of T cell tolerance and to effectively activate both T cells and B cells (49).

These results support the idea that it may be possible to exploit orally induced tolerance in the treatment of human disease such as allergy or autoimmunity. Preexisting IgE responses and delayed hypersensitivity have been successfully down-regulated by Ag feeding in experimental animals, thus providing experimental support to this notion (50, 51). In regard to allergy, hyporesponsiveness to allergens such as tree or grass pollens and leaf extracts has been induced with variable success (52, 53). The induction of T cell tolerance to ingested Ags may be of considerable importance in the amelioration of diseases in which T cells represent the dominant effector mechanism. As mentioned above, oral tolerance to ingested autoantigens has been effectively used in experimental autoimmune diseases (7–13). Whether the feeding of autoantigens to humans can suppress the further progression of autoimmune disease remains to be demonstrated, but clinical studies are under way in multiple sclerosis (54) and rheumatoid arthritis (55). If this can be achieved, the induction of T cell tolerance by Ag feeding may represent a novel form of treatment for autoimmune diseases and hypersensitivity disorders.

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INDUCTION OF IMMUNE TOLERANCE TO HUMAN TYPE I COLLAGEN IN PATIENTS WITH SYSTEMIC SCLEROSIS BY ORAL ADMINISTRATION OF BOVINE TYPE I COLLAGEN

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Objective. **To determine whether oral tolerance to type I collagen (CI) could be induced in patients with systemic sclerosis (SSc).**

Methods. **Twenty adult patients with limited or diffuse SSc were enrolled in a study to receive 0.1 mg of solubilized native bovine CI daily for 1 month, followed by 0.5 mg daily for 11 months. Peripheral blood mononuclear cells (PBMC) were obtained from the patients and cultured with human α1(I) and α2(I) chains, before and after CI treatment. Culture supernatants were analyzed for levels of interferon-γ (IFNγ) and interleukin-10 (IL-10). Sera obtained before and after treatment were analyzed for levels of soluble IL-2 receptor (sIL-2R). Although this study was not intended to assess the clinical efficacy of oral CI administration in SSc, selected measures of disease severity and organ involvement were evaluated.**

Results. **Oral administration of CI to SSc patients induced significant reductions in levels of IFNγ and IL-10 in α1(I)- and α2(I)-stimulated PBMC culture supernatants, indicating that T cell immunity to CI was decreased by this treatment. Serum levels of sIL-2R also decreased significantly after oral CI treatment, suggesting a reduction in T cell activation. Significant improvements occurred in the modified Rodnan skin thickness score and the modified Health Assessment Questionnaire after 12 months of oral CI in this open trial. The lung carbon monoxide diffusing capacity improved statistically and showed a trend toward clinically significant improvement.**

Conclusion. **Oral administration of bovine CI to patients with diffuse or limited SSc induces a reduction in T cell reactivity to human CI, appears to be well tolerated, and does not worsen the disease. Further evaluation of oral tolerance to CI in patients with SSc is justified to determine whether it has therapeutic efficacy.**

Type I collagen (CI) is the most abundant of all collagens in humans (1). It is present in blood vessels, skin, lungs, heart, kidneys, and intestines, all of which are affected in systemic sclerosis (SSc) (1). CI is a heterotrimer molecule composed of two identical α1(I) chains and one α2(I) chain (1). Each α chain contains 1,014 amino acid residues (1). Human and bovine CI have ~92% homology at the amino acid level (2,3). Evidence for cellular immunity to CI in SSc patients was first demonstrated by our group in 1976 (4) and has been confirmed by other investigators (5,6). We found that peripheral blood mononuclear cells (PBMC) from 92% of SSc patients produce chemotactic cytokines when cultured with CI, whereas only 8% of PBMC from healthy subjects do so (4). Hawrylko et al (5) also showed that peripheral blood CD4+ T cells from patients with SSc produce interleukin-2 (IL-2) in a dose-dependent manner in response to stimulation with human CI, while those from healthy subjects do not.

A major portion (approximately one-third) of the body's immune cells reside in the gut-associated lymphoid tissue (GALT) (7). The GALT is particularly effective in mounting a tolerogenic response to ingested soluble proteins (7,8). This process, called oral toler-

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ance, has been repeatedly demonstrated in laboratory animals. For example, when mouse strains susceptible to experimental allergic encephalomyelitis (EAE) after systemic immunization with myelin basic protein (MBP) are fed MBP prior to immunization, they develop less EAE or no EAE compared with placebo-fed MBP-immunized controls (9).

The mechanisms that mediate oral tolerance include active cellular suppression (regulatory T cells), clonal anergy, and clonal deletion (10–12). The particular dose of antigen and the frequency of feeding determine which mechanism(s) predominates (10,12). Multiple oral feedings of low-dose soluble antigen favor development of regulatory CD4+ T cells that secrete Th2 cytokines, such as IL-4 and IL-10, and transforming growth factor β1 (TGFβ1)–secreting T cells (Th3 cells) (10,12). These regulatory T cells migrate to peripheral sites throughout the body, and when they encounter the antigen to which they are tolerized, they collectively secrete IL-4, IL-10, and TGFβ1, which can down-regulate Th1 CD4 cells reacting to a variety of antigens, a process called "bystander suppression" (10,13,14).

Since many of the antigens that are involved in human autoimmune diseases are unknown, it is theoretically possible that, by feeding low doses of antigen from the organs or tissues that are the target of autoimmune attack, T cell responses to other autoantigens perpetuating the disease can be down-regulated. CI qualifies as a candidate oral tolerance antigen in SSc, in that it is present in all of the target organs. Since most SSc patients exhibit sensitization to CI (1,4–6), as manifested by cytokine production by PBMC during culture with CI or constituent α1 and α2 chains, successful tolerization to CI after it has been orally administered to SSc patients can be assessed by determining whether there are decreases in cytokine production by PBMC cultured with CI α chains. The present phase I study was undertaken to determine whether daily administration of oral bovine CI to patients with SSc would result in down-regulation of the immune response to human CI.

PATIENTS AND METHODS

Patient recruitment and characteristics. This study was approved by the Institutional Review Board at The University of Tennessee Health Science Center. Patients were recruited from University of Tennessee and community rheumatology practices in Memphis. Inclusion criteria were as follows: age ≥18 years; diagnosis of limited or diffuse SSc by the criteria of the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) (15); and patient's PBMC demonstrated reactivity to bovine CI, as defined by the production of IL-10 (≥2 times baseline production) when cultured with native bovine CI. Patients taking D-penicillamine, captopril, or calcium channel blockers were required to be receiving stable doses of these agents for at least 3 months prior to enrollment. The maximum allowable dosage for D-penicillamine was 750 mg/day. Patients taking corticosteroids were required to be receiving a stable dose for at least 1 month prior to enrollment; the maximum allowable dosage was 10 mg/day of prednisone equivalent.

Patients were excluded from the study for the following reasons: inability to render an informed consent in accordance with institutional guidelines; receiving another investigational drug (excluding D-penicillamine) within 90 days of study initiation; a concurrent serious medical condition that, in the opinion of the investigators, made the patient inappropriate for the study; an SSc-like illness associated with environmental, ingested, or injected agents, such as L-tryptophan, tainted rapeseed oil, vinyl chloride, or bleomycin; morphea, linear scleroderma, or eosinophilic fasciitis; a positive pregnancy test; use in the previous 3 months of cyclophosphamide, cyclosporin A, methotrexate, or azathioprine; allergy to beef; or malabsorption syndrome.

Design and duration of the study. The study was an open-label trial to determine whether oral CI treatment would down-regulate PBMC cytokine production when cultured with α1(I) and α2(I). Patients received 0.1 mg/day of solubilized bovine CI for 1 month, followed by 0.5 mg/day for 11 months. Collagen was solubilized in 0.1*M* acetic acid and aliquoted into individual-dose vials. Patients kept the vials refrigerated. Each morning, the patient added 1 vial of the CI preparation to 4–6 ounces of cold orange juice and drank it just before eating breakfast. Patient compliance was monitored by counting the numbers of empty and full vials returned at each visit.

Concomitant medication. Patients were not allowed to increase dosages of D-penicillamine, captopril, calcium channel blockers, or corticosteroids during the study. Patients were dropped from the study if increases in any of these medications were deemed medically necessary by their primary physicians.

Clinical measurements. Significant clinical responses were not expected due to the small study size and the variability in disease classification, manifestations, and duration. However, the following measures of disease severity and organ involvement were evaluated: modified Rodnan skin thickness scores (MRSS) (16) at 0, 1, 2, 3, 6, 9, and 12 months; pulmonary function tests (PFTs; spirometry) with measurement of the diffusing capacity for carbon monoxide (DLco; performed by the same personnel using the same equipment) at 0, 3, 6, 9, and 12 months; serum creatinine levels at 0, 1, 3, 6, 9, and 12 months; and the modified Health Assessment Questionnaire (M-HAQ) (17,18) at 0, 3, 6, 9 and 12 months.

Microculture of SSc PBMC with α1(I) and α2(I). Briefly, before and after 3, 6, and 12 months of oral bovine CI treatment, PBMC were isolated by Ficoll-Hypaque density-gradient centrifugation and set up in culture in 48-well tissue culture plates (2×10^6 cells in 0.5 ml of RPMI 1640 containing 100 units/ml of penicillin, 100 μg/ml of streptomycin, 2 m*M* L-glutamine, 20 m*M* HEPES, and 5% fetal calf serum). Cultures were set up with 50 μg/ml each of purified bovine α1(I) and α2(I) chains, and phytohemagglutinin (PHA; 10 μg/ml) and phosphate buffered saline (PBS) as controls in duplicate wells. After 5 days of culture, supernatants from duplicate

wells were pooled, harvested by centrifugation, and frozen at −70°C until assayed for cytokine levels (within 30 days).

Measurement of cytokines in serum and PBMC supernatants. After screening for several cytokines in supernatants from SSc PBMC cultured with α1(I) and α2(I), we found that most SSc patients produced increased amounts of interferon-γ (IFNγ) and IL-10 protein, as measured by enzyme-linked immunosorbent assay (ELISA). These cytokines were subsequently measured in all culture supernatants.

IFNγ and IL-10 levels were measured by commercial ELISA (R&D Systems, Minneapolis, MN) in supernatants harvested from microcultures of SSc PBMC stimulated by PHA, α1(I), and α2(I), and PBMC plus PBS as a control for background cytokine production. A positive response to α1(I) or α2(I) was arbitrarily defined as IL-10 or IFNγ levels in α1(I)- or α2(I)-stimulated PBMC culture supernatants that were ≥2 times the respective cytokine level in the PBMC plus PBS control supernatant. Soluble IL-2 receptor (sIL-2R) levels were measured by ELISA (R&D Systems) in sera obtained before and after 12 months of oral CI treatment. All samples were tested in duplicate.

Measurement of T cell subsets by flow cytometry. Isolated PBMC obtained from samples taken at 0 and 6 months of oral CI treatment were reacted with a panel of monoclonal antibodies that recognize T cell–specific markers CD4+, CD8+, CD3+DR+, CD4+CD45+RA+, CD4+CD45+R0+, CD8+CD45+RA+, CD8+CD45+RO+, and CD4+CD26+ and analyzed by fluorescence-activated cell sorter at the University of Tennessee Molecular Resource Center.

Preparation and handling of bovine CI. Bovine CI was prepared as previously described (19). Bovine fetuses from pregnant cows were obtained from a local slaughterhouse within 1 hour of death. The skins of 4 fetal calves were removed and maintained at 4°C throughout the preparation. The tissue was sliced into strips and processed through a household meat grinder, then homogenized in a Waring blender with ice chips. The homogenate was centrifuged (10,000*g*) for 30 minutes and reextracted twice with 1*M* NaCl (pH 7.6, with 0.05*M* Tris HCl) and twice with 0.1*M* acetic acid to remove some type III soluble collagen and much of the noncollagenous components. The final pellet (~500 gm) was suspended in 16 liters of 0.1*M* acetic acid, and the pH was adjusted to 2.8 with formic acid.

Type I collagen was solubilized by overnight (16 hours) digestion with 20 gm of pepsin (3× crystallized; Sigma, St. Louis, MO) at 4°C. The digest was centrifuged (10,000*g* for 30 minutes), and the insoluble pellet was discarded. Type I collagen in the supernatant was precipitated by addition of 5*M* NaCl solution to a final concentration of 0.8*M*. This was centrifuged as before, and the pellet was redissolved in 0.1*M* acetic acid. The pH was adjusted to 7.4 with 0.05*M* Tris and 10*M* NaOH to inactivate pepsin. Solid NaCl was added to a concentration of 1*M*, and the solution was centrifuged. The supernatant was collected, and the NaCl content was increased to 1.7*M* with 5*M* NaCl. This was centrifuged to remove contaminating CIII. The 1.7*M* NaCl supernatant was further adjusted to 2.5*M* NaCl, which precipitated the CI.

The CI pellet was collected by centrifugation and redissolved in 0.5*M* NaCl, 0.05*M* Tris, diluted to 0.2*M* NaCl with water, and 50 gm of DE-52 was added to create a slurry. This was stirred overnight and centrifuged to remove any DEAE that had bound any remaining pepsin and contaminating glycosaminoglycans. The supernatant was dialyzed against 0.02*M* NaH_2PO_4 to precipitate CI. The pellet was redissolved in 0.01*M* acetic acid, dialyzed exhaustively against the same, and stored at −80°C until used.

The homogeneity of the CI was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, which showed an α1(I)/α2(I) ratio of 2:1 with no contaminating type V or type III collagen. The α1(I) and α2(I) collagen chains were separated by carboxymethyl cellulose chromatography, and constituent α chains were digested with cyanogen bromide (19).

Frozen CI stock containers were allowed to thaw over 2–3 days at 4°C prior to dispensing into vials. Thawed collagen was centrifuged at 4°C at 12,000*g* to remove particulates. The collagen was diluted to a concentration of 50 μg/ml or 250 μg/ml with cold (4°C) 0.1*M* acetic acid. The diluted CI was filtered at 4°C through a glass fiber Acrodisc (Gelman Sciences, Ann Arbor, MI) and then a 0.45μ filter (Nalgene filter; Nalge, Rochester, NY) and aliquoted (2 ml) into sterile 2-ml screw-top polypropylene vials (Nalgene vials; Nalge). Vials were placed in plastic bags (35 vials/bag) and stored frozen at −20°C until given to the patients.

Statistical analysis. Cytokines produced by PBMC in response to culture with bovine CI, serum levels of sIL-2R, results of PFTs, and clinical variables were analyzed by Student's paired *t*-test to determine whether significant changes occurred after 3, 6, 9, or 12 months of oral CI treatment, compared with pretreatment values. Correlations of the M-HAQ or the MRSS versus IL-10 or IFNγ were analyzed by Spearman's correlation test.

RESULTS

Patient characteristics at study entry. Twenty-five patients with SSc were screened. Twenty-four exhibited production of IL-10 or IFNγ that was ≥2-fold higher than the levels in parallel cultures of the patients' PBMC plus PBS but without α1(I) or α2(I). Four patients had complications of SSc or other medical diseases that disqualified them from the study. One patient was enrolled but withdrew from the study very early. The characteristics of the remaining 19 patients are shown in Table 1.

The patients were predominantly female, and the majority had late, diffuse disease. All patients satisfied the ACR preliminary criteria for the classification of SSc. Thirteen were white and 6 were African American. Only 3 patients had a disease duration of <2 years. Only 3 patients were currently taking D-penicillamine. The 5 patients taking nonsteroidal antiinflammatory drugs (NSAIDs) discontinued these during the last 6 months of CI treatment. Three patients took 5 or 10 mg/day of prednisone throughout the study period.

Side effects, withdrawals, and compliance. Seventeen patients were treated for 12 months. Two patients dropped out because of difficulty with transportation: one

Table 1. Characteristics of the patients taking oral type I collagen for 1–12 months*

Sex	
Female	15
Male	4
Race	
White	13
African American	6
SSc type	
Diffuse SSc	14
Limited SSc	5
Age, mean ± SD years	50.7 ± 2.6
Disease duration	
Mean ± SD years	9.1 ± 2.0
<2 years' duration	3
Medication use	
Penicillamine	3
NSAIDs	5
Prednisone	3

* Except as noted otherwise, values are the number of patients. SSc = systemic sclerosis; NSAIDs = nonsteroidal antiinflammatory drugs.

very early (<1 month), the other after 6 months. One patient developed a foot drop of uncertain etiology and was removed from the study after 6 months of therapy. No other possible side effects were noted. There was 100% compliance by each patient until the time each dropped out of the study or the study was completed.

Induction of T cell tolerance to CI by administration of oral CI. The daily administration of bovine CI for 12 months was accompanied by significant reductions in IFNγ production by PBMC cultured with purified α1(I) and α2(I) chains of human CI as measured after 6 and 12 months of treatment (Figure 1A). IFNγ is a Th1 cytokine, and its reduced production by α1(I)- and α2(I)-stimulated PBMC suggests that oral tolerance to CI was effected. Quite surprisingly, IL-10 levels in the same PBMC culture supernatants were also significantly reduced after 3, 6, and 12 months of oral CI treatment (Figure 1B). The production of IFNγ and IL-10 by PBMC stimulated with PHA was not statistically different before or at 3, 6, or 12 months after oral CI treatment (results not shown).

After 12 months of oral CI treatment, there was a significant reduction (as determined by Student's paired *t*-test) in the serum levels of sIL-2R (Figure 2).

T cell subsets measured by the following markers did not change after treatment with oral CI treatment: CD8+, CD4+, CD3+DR+, CD4+CD45+RA+, CD4+CD45+R0+, CD4+CD26+, CD8+CD45+RA+, and CD8+CD45+RO+ (results not shown).

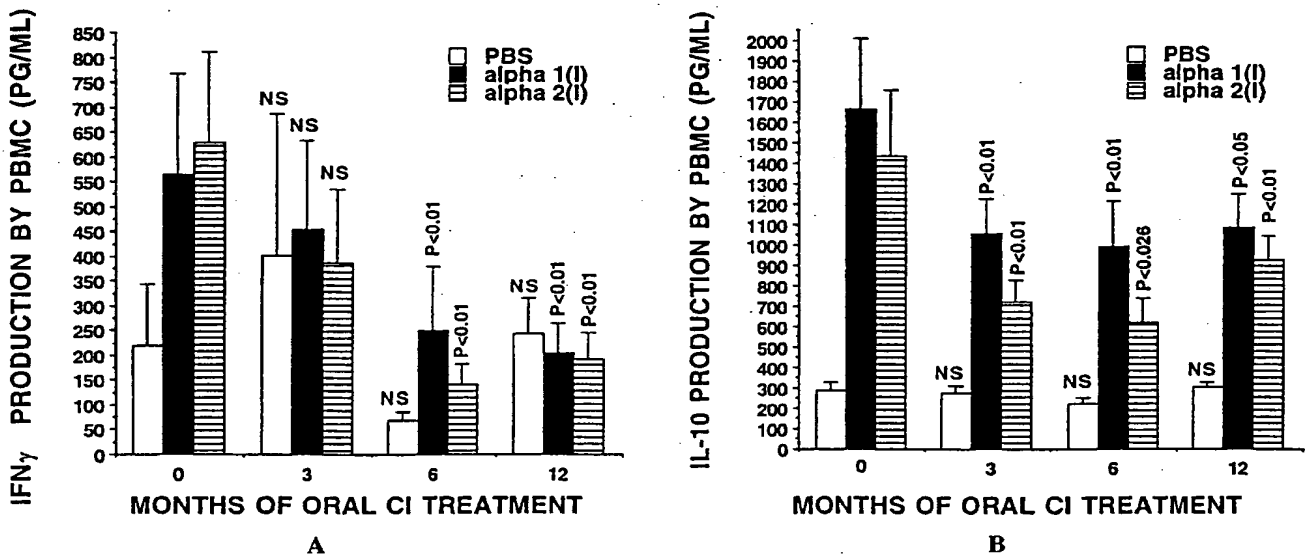

Figure 1. Production of A, interferon-γ (IFNγ) and B, interleukin-10 (IL-10) by peripheral blood mononuclear cells (PBMC) from patients with systemic sclerosis. PBMC were cultured with α1(I) and α2(I) chains before and after 3, 6, and 12 months of oral treatment with bovine type I collagen (CI; 500 μg/day). Harvested culture supernatants were analyzed for levels of IFNγ and IL-10 by commercial enzyme-linked immunosorbent assay, as described in Patients and Methods. Values are the mean and SEM. *P* values determined by Student's paired *t*-test; NS = not significant.

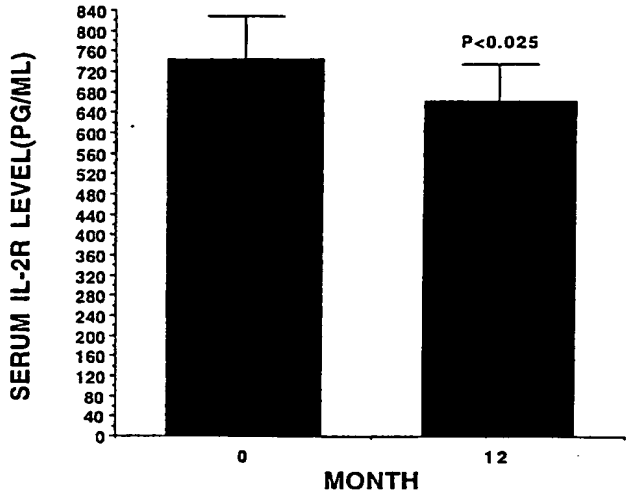

Figure 2. Serum levels of soluble interleukin-2 receptor (IL-2R) in patients with systemic sclerosis. Sera were obtained before and after 12 months of oral treatment with bovine type I collagen and analyzed by commercial enzyme-linked immunosorbent assay for soluble IL-2R levels. Values are the mean and SEM of the 17 patients completing 12 months of treatment. *P* value determined by Student's paired *t*-test.

Improvement in clinical variables. The M-HAQ difficulty in performing activities of daily living (ADL) scale and the MRSS were significantly improved after 6 and 12 months in this open-label study (Figures 3A and B). After 12 months of CI treatment, the M-HAQ ADL difficulty scale had improved 27%, from a baseline value of 0.66 ± 0.14 (mean ± SEM) to a value of 0.48 ± 0.14 ($P < 0.05$). The MRSS declined steadily, and after 12 months of CI treatment, had decreased by 23%, from a baseline value of 26.35 ± 2.35 to a value of 20.29 ± 2.53 ($P < 0.005$) (Figure 3B). In the patients with diffuse SSc, the MRSS decreased by 26.6% after 12 months of CI treatment, from a baseline value of 28.6 ± 2.5 to a value of 21.0 ± 2.7 ($P < 0.005$) (results not shown). There were no significant correlations between MRSS score or M-HAQ score and decreases in IL-10 or IFNγ production by PBMC cultured with CI α chains after 12 months of CI treatment (results not shown).

Because of patient noncompliance and scheduling problems, only 11 of the 17 patients who finished the study had DLco measurements and PFTs performed at 0 and 12 months. For these 11 patients, the mean DLco, corrected for alveolar volume and hemoglobin, increased by 9.58% from 3.34 to 3.66 ml/minute/mm Hg (*P*

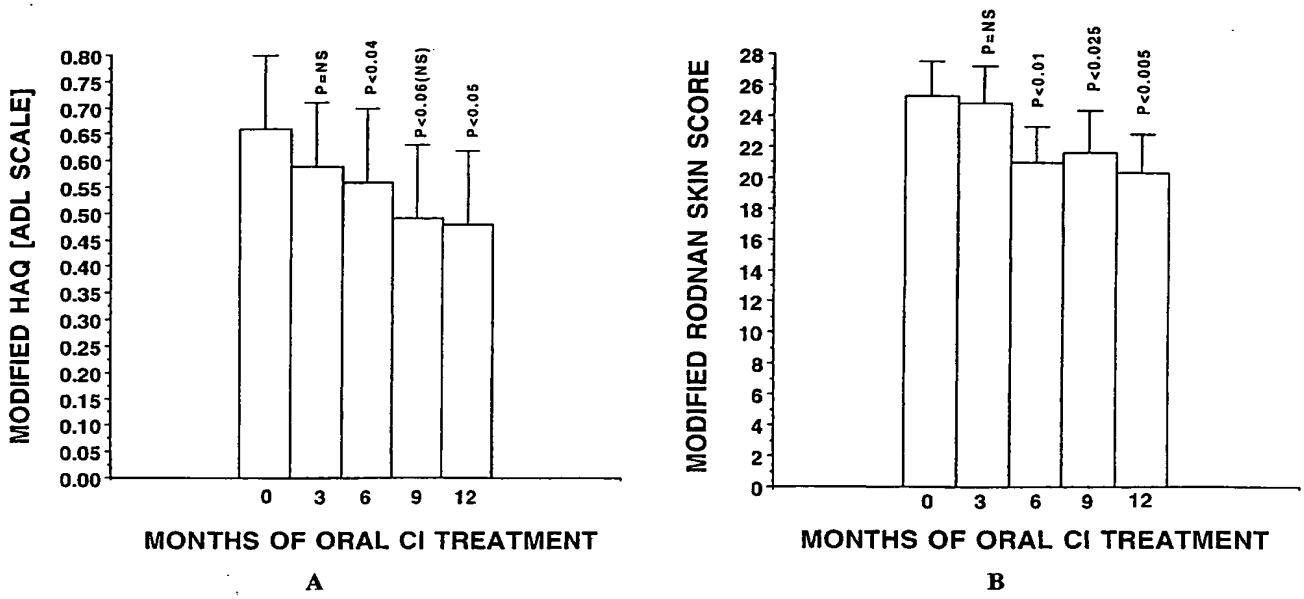

Figure 3. A, The modified Rodnan skin thickness score (MRSS) and B, the modified Health Assessment Questionnaire (M-HAQ) scores in patients with systemic sclerosis. The MRSS and the M-HAQ were measured before and after 12 months of oral treatment with bovine type I collagen (CI). Values are the mean and SEM. *P* values determined by Student's paired *t*-test; NS = not significant. ADL = activities of daily living.

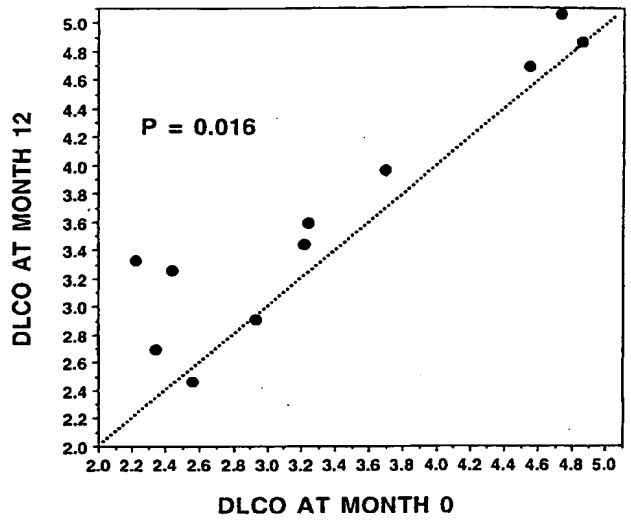

Figure 4. Diffusing capacity for carbon monoxide (DLCO) in 11 patients with systemic sclerosis, before and after 12 months of oral treatment with bovine type I collagen. The dotted line is a reference line with a slope of 1. *P* value determined by Student's paired *t*-test.

< 0.016) after 12 months of CI therapy (Figure 4). There was no significant change in forced vital capacity or in serum creatinine values.

DISCUSSION

In patients with limited and diffuse SSc, the administration of oral bovine CI for 1 month at a dosage of 100 μg/day and for 11 months at 500 μg/day resulted in significant reductions in IFNγ and IL-10 production by CI α-chain–stimulated PBMC. Levels of serum sIL-2R were also significantly reduced after induction of oral tolerance to CI. This reduction in sIL-2R, although small, suggests that overall T cell stimulation was reduced. To our knowledge, levels of sIL-2R in sera have not been reported in other studies of human oral tolerance or in animal models of oral tolerance. Therefore, the significance of this modest reduction in serum sIL-2R levels in the context of oral tolerance is not known at present. Taken together, these data indicate that oral tolerance to CI was effected by this treatment regimen.

The mechanism(s) by which this oral CI regimen induced these immune changes is not readily apparent. It is likely that IFNγ production by CI α-chain–stimulated PBMC is largely from CD4+ Th1 cells, but natural killer cells are also a potential source of this cytokine. The reduced production of IFNγ by PBMC CD4+ T cells could be due to 1 or a combination of the 3 mechanisms of oral tolerance induction (i.e., suppressive regulatory T cells, clonal anergy, or clonal deletion) (10–12).

The reduced IL-10 production by SSc CI α-chain–stimulated PBMC after oral CI treatment was unexpected, given that IL-10 has been reported to be up-regulated in peripheral lymphoid tissue or in target organs in autoimmune immune models after oral tolerance induction by low-dose antigen (20). However, in some circumstances, IL-10 can be produced by Th1 cells, and in humans, there is less rigidity to the Th1/Th2 paradigm originally described using clonal mouse T cells (20–22). In addition, IL-10 is produced by cells other than CD4+ T cells (23,24). Monocyte/macrophages are a major source of this cytokine (23,25). The reduced IL-10 production by CI α-chain–stimulated SSc PBMC after oral CI treatment may reflect overall decreased T cell responsiveness to the α chains, and therefore decreased stimulation to monocytes by IFNγ or other cytokines from T cells that up-regulate IL-10 production by monocytes. Finally, published studies of oral tolerance in animal models have not measured antigen-stimulated PBMC production of IL-10, or other cytokines elaborated by PBMC, before and after oral tolerance induction. The published studies of animal models of oral tolerance all measure cytokine expression in lymphoid tissue or target organs rather than peripheral blood.

NSAIDs are known to inhibit oral tolerance in animal models and may be a confounding factor in human oral tolerance (26–28). For this reason, we advised the 5 patients who had been taking NSAIDs for the first 6 months of the trial to discontinue them for the last 6 months of the trial, which they did.

Although there were significant improvements in the MRSS and M-HAQ scores, these findings should be viewed with caution, since this was an open-label study and these changes may reflect variations or spontaneous changes in the disease or a placebo effect. The DLco values, while showing statistically significant improvement, are still just below the clinically significant cutoff of $\geq 10\%$. Clearly, a larger population of more homogeneous patients with diffuse SSc needs to be evaluated in a randomized, double-blind, placebo-controlled study before it can be categorically concluded that oral CI tolerance induction ameliorates the SSc disease process.

One could speculate that the mechanism by which oral CI might possibly ameliorate SSc could involve anergy and/or suppression depending on the

dose of CI given. Although the 500 μg/day dosage of CI induced oral tolerance, clearly other dosages need to be studied. The feeding of CI to SSc patients could anergize autoreactive cells and/or generate major histocompatibility complex class I– or class II–restricted regulatory T cells that sequester in involved tissues, where they release small amounts of immunosuppressive cytokines (IL-4, IL-10, TGFβ1) that down-regulate autoaggressive cells by the mechanism of antigen-driven bystander suppression. By antigen-driven bystander suppression, these CI-specific T cells could down-regulate T cell interactions with other antigens (29), as has been demonstrated in autoimmune animal models in which oral tolerance has been induced by oral administration of antigens from organs that are the target of attack.

These animal models provide a theoretical basis for predicting that in SSc patients, CI, although it may not be an initiating antigen of SSc or even be involved in its pathogenesis, when given as an oral tolerogen, may well suppress T cell–mediated fibrogenesis by suppressing activated T cells. If activated CD4+ T cells present in the tissues of SSc patients could be down-regulated, then with time, the fibrogenic phenotype of SSc fibroblasts might revert to normal. It is known that after serial passage of SSc fibroblasts in vitro for several generations, they regain a more normal phenotype with regard to matrix synthesis (30). Also, patients with longstanding SSc tend to have less skin thickening and collagen deposition than they had in earlier stages of their disease.

While IL-4 and TGFβ at high concentrations (~50 ng/ml and 5 ng/ml, respectively) can up-regulate collagen synthesis by cultured fibroblasts in vitro, lower concentrations (e.g., TGFβ1 at 1,000 times less) are capable of modulating immune cells (30–34). The fact that oral CI treatment did not increase skin or lung fibrosis suggests that if GALT-derived regulatory T cells producing these cytokines were generated by oral CI treatment, the levels of these cytokines are likely to be lower than is required to trigger collagen synthesis by fibroblasts.

Oral CI administration appears to be safe in SSc patients. Its efficacy needs to be assessed by a larger placebo-controlled, double-blind trial.

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Errata

In the article by García-Porrúa et al published in the March 2000 issue of *Arthritis & Rheumatism* (pp 584–592), there was an error in the second full sentence in the left column on page 589. The sentence should have read, "Six of 39 patients diagnosed as having idiopathic EN (15.4%) had 1 or more predictive factors for *secondary* EN, and 64 of 67 patients diagnosed as having secondary EN (95.5%) had 1 or more predictive factors for secondary EN" [emphasis added]. This is also how the first sentence of the first footnote in Table 4 should have read.

In the article by van der Heijden et al in the March 2000 issue (pp 593–598), the reference cited at the end of the first sentence in the second paragraph of Patients and Methods (page 594) should have been reference 11, rather than reference 4. The reference cited at the end of line 12 in the second paragraph of the Discussion (page 597) should also have been reference 11, rather than reference 8.

We regret the errors.